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Metabolic Engineering of *Pseudomonas putida* for Methylmalonyl-CoA Biosynthesis to Enable Complex Heterologous Secondary Metabolite Formation

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Summary

An operon consisting of three open reading frames, annotated in silico as methylmalonyl-CoA (mm-CoA) epimerase, mm-CoA mutase (MCM), and meaB, was identified in the sequencing project of the myxobacterium *Sorangium cellulosum* So ce56. This putative MCM pathway operon was subcloned from a bacterial artificial chromosome by Red/ET recombineering onto a minimal replicon derived from p15A. This plasmid was modified for integration and heterologous expression in *Pseudomonas putida* to enable the production of complex secondary metabolites requiring mm-CoA as precursor. Methylmalonate was identified in the recombinant *P. putida* strain by an analysis method based on gas chromatography/mass spectrometry. The engineered strain is able to synthesize polyketides requiring mm-CoA as an extender unit, which was demonstrated by the production of myxothiazol after integration of the biosynthetic gene cluster into the chromosome, followed by induction of expression.

Introduction

The variety and number of metabolites produced in the natural world is astonishing. Over 100,000 secondary metabolites with a molecular weight less than 2,500 Da have been described, mainly isolated from microbes and plants [1]. Natural products are synthesized starting from small molecules by multiple enzymatic steps [2] and classified according to the building blocks used to build up the metabolite (e.g., polyketides or nonribosomally synthesized peptides). The polyketides are synthesized from acyl-CoA precursors by polyketide synthases (PKS) in a reaction scheme that is related to fatty acid biosynthesis. Nonribosomally generated peptides are assembled by nonribosomal peptide synthetases (NRPS) with proteinogenic and unusual amino acids, as well as α-hydoxy and carboxylic acids [3]. Natural products are significant because they often exhibit biological activities, such as antibiotics, cytostatics, insecticidals or antifungals [4]. The problem of emerging resistance emphasizes the need to search for new drugs. Within this process, the discovery and exploitation of new resources is considered important [5]. One possibility is the activation of "silent" biosynthetic gene clusters revealed by microbial genome projects [5]. Even well-known producers of secondary metabolites, such as *Streptomyces coelicolor* A3(2) or *S. avermitilis*, harbor many more biosynthetic gene clusters than anticipated [6, 7]. Another resource might be metagenomes, which represent the genetic information of a specific location at a certain point in time for all microorganisms, including those not cultivatable [5]. A further possibility is the mining of microorganisms from ecological niches [8]. Searching for new activities in these sources, particularly in metagenomes, depends upon well-behaved and well-characterized heterologous expression systems. There are several examples of heterologous expression of biosynthetic pathways from microorganisms in closely related genera [5, 9]. Heterologous expression of secondary metabolites in unrelated hosts is also manageable. For example, *Escherichia coli* was genetically modified in several steps to produce 6-deoxyerythronolide (6-dEB), the aglycone of the antibiotic erythromycin [10]. More recently, we used *P. putida* KT2440 to express the myxobacterial myxochromide PKS/NRPS hybrid gene cluster [11]. The production was possible because *P. putida* provided all the building blocks for the biosynthesis of myxochromide, including a broad-spectrum phosphopantetheinyl transferase capable of posttranslational activation of the carrier domains in the PKS/NRPS hybrid [12].

Many polyketides require methylmalonyl-CoA (mm-CoA) as an extender unit for the incorporation of C3 units. mm-CoA can be synthesized in two ways: first, through the action of propionyl-CoA carboxylase (PCC); and, second, in the reverse reaction of the mm-CoA mutase (MCM) and epimerase pathway from succinyl-CoA (Figure 1). Propionyl-CoA is a central metabolite in the degradation of the amino acids L-met, L-ile and L-val. It is also the end product in the degradation of uneven fatty acids by β-oxidation. According to biochemistry textbooks, propionyl-CoA is carboxylated to (2S)-mm-CoA by the biotin-dependent PCC. The racemization of (2S)-mm-CoA into its (2R)-stereoisomer is catalyzed by mm-CoA epimerase. (2R)-mm-CoA is then the substrate for the cobalamine-dependent MCM in the formation of succinyl-CoA, which is part of the Krebs cycle (Figure 1) [13, 14]. mm-CoA was not detected in pseudomonads

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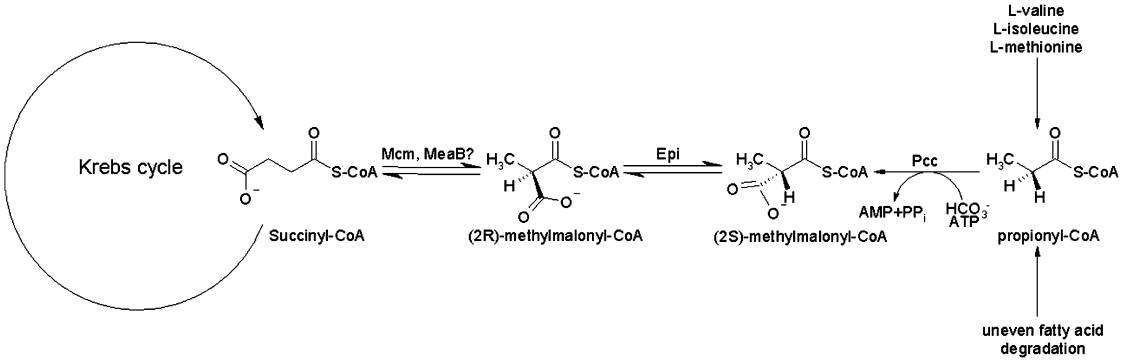

Figure 1. Propionyl-CoA Metabolism/MCM Pathway

MCM, mm-CoA mutase; MeaB, mm-CoA mutase enzyme complex protecting protein; Epi, mm-CoA epimerase; Pcc, propionyl-CoA carboxylase.

[15], although they can grow with L-val and L-ile as sole carbon source (Figure 1). As mm-CoA is not made in *P. putida* in detectable levels, metabolic engineering is required to supply the necessary extender units for biosynthesis of mm-CoA-dependent secondary metabolites.

Myxobacteria are a prominent resource of secondary metabolites [16, 17], and genetic studies on the biosynthesis of the respective compounds are emerging [18]. It can be assumed that myxobacteria harbor genes responsible for the formation of mm-CoA, because of their capacity to produce polyketide-type secondary metabolites with mm-CoA as extender units (e.g., epothilone [19–21], soraphen [16, 22, 23], or myxothiazol [24, 25]).

In this study, we report the identification of an operon encoding an mm-CoA epimerase (EC 5.1.99.1), MCM (EC 5.4.99.2), and an enzyme complex-stabilizing factor, MeaB, in the ongoing genome sequencing project of the myxobacterium *Sorangium cellulosum* So ce56 (Figure 1). We report the heterologous expression of this operon in *P. putida*, KT2440, and the identification of mm-CoA in the recombinant *Pseudomonas* strain, by a new isotope dilution analysis method based on gas chromatography coupled with mass spectrometry. mm-CoA enabled the heterologous production of the myxobacterial secondary metabolite, myxothiazol, in this engineered strain.

Results

Identification of the mm-CoA operon in *S. cellulosum*

An operon consisting of three open reading frames (ORFs) was identified in the genome of *S. cellulosum* So ce56 when MCM and mm-CoA epimerase from *Propionibacterium freudenreichii shermanii* were used as query in BLASTP [26] searches. The third ORF (*meaB*), which exhibits similarity to lysine/arginine/ornithine (LAO) transport proteins, is located downstream of the mutase gene. Such proteins are conserved neighbors of prokaryotic *mcm* genes and are, therefore, considered to be involved in propionyl-CoA metabolism [27]. The arrangement of the operon is depicted in Figure 2A, and an alignment of the predicted MCM is shown in Figure 2B.

The predicted mm-CoA epimerase (*epi*) is 519 nucleotides long, with no clearly identifiable ribosome binding site (RBS) in the immediate vicinity upstream of the proposed ATG start codon, and the deduced protein (172 amino acids) shows greatest similarity to predicted glyoxylases/bleomycin resistance genes (lactoglutathione [LGSH] lyase family) from *Solibacter usitatus* Ellin6076 (ZP_00519667; identities: 98/149 [65%]; positives: 125/149 [83%]), *Nocardioides* sp. JS614 (ZP_00656876; identities: 58/151 [38%]; positives: 87/151 [57%]), and to a predicted mm-CoA epimerase from *Geobacter sulfurreducens* PCA (NP_954343; identities 59/139 [42%]; positives: 83/139 [59%]). The coding sequences of the predicted epimerase and mutase overlap for nine nucleotides (…CATGGATGAC…), indicating a coupled translation.

The ORF encoding the predicted MCM (*mcm*) is 2649 nucleotides in size, and a putative RBS (GAGG) can be found 12 nucleotides upstream of the proposed ATG start codon, with no alternative start codon in the immediate vicinity. The deduced protein (882 amino acids, 95 kDa) shows greatest similarities to the MCMs of *Chloroflexus aurantiacus* (ZP_00358667; identities: 460/670 [68%]; positives: 537/670 [80%]) and *Leptospira interrogans* serovar Copenhageni str. Fiocruz L1-130 (YP_003598; identities: 460/677 [67%]; positives: 539/677 [79%]). All known MCMs function as dimers. The genes for MCM from *P. freudenreicheii shermanii* [28], *Streptomyces cinnamonensis* [29], *E. coli* [30], humans [31], and mice [32] have been cloned and sequenced. The enzymes from *P. freudenreichii shermanii* and *S. cinnamonensis* are heterodimers of two homologous subunits: α subunit (~79 kDa) and β subunit (~65 kDa) [28, 29]. The other three enzymes are homodimers (~79 kDa) [30–32]. No second subunit could be identified in the *S. cellulosum* So ce56 genome, which suggests that the active protein is a homodimer. It carries an N-terminal tail of approximately 150–200 amino acids, which shows approximately 30% identity with a part of the small subunits of other MCMs (data not shown). This homology is not detected when the whole peptide is used as input sequence in the BLASTP search; alignments then start around amino acid 200 of the sequence. An alignment of MCM with some other selected MCMs is shown in Figure 2B.

The ORF of *meaB* is 993 nucleotides in size, and a putative RBS (GAGG) is located 9 nucleotides upstream of the proposed TTG start codon. The stop

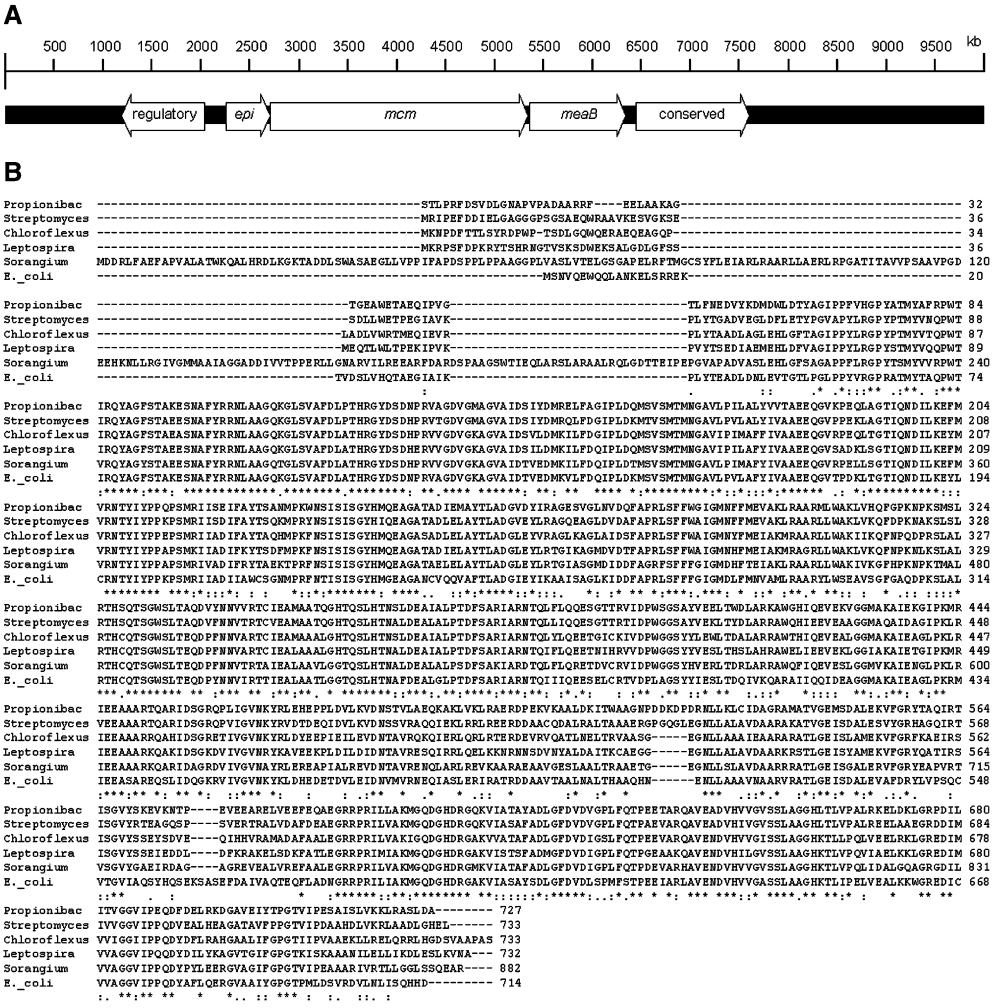

Figure 2. The MCM Pathway and Alignment of MCMs

(A) Methylmalonyl-CoA mutase pathway—genetic organization in the *S. cellulosum* So ce56 genome. *epi*, methylmalonyl-CoA epimerase; *mcm*, methylmalonyl-CoA mutase; *meaB*, complex stabilizing protein; regulatory, regulatory protein; conserved, conserved hypothetical protein

(B) Aligment of selected MCM performed with ClustalW. Propionibac, *P. freudenreichii shermanii*, α-subunit (P11653); Streptomyces, *Streptomyces cinneamonensis*, α-subunit (Q05065); Chloroflexus, *Chloroflexus aurantiacus* (Q3DXY6); Leptospira, *Leptospira interrogans* sv *copenhageni* (Q75FI0); Sorangium, *S. cellulosum* So ce56; *E. coli*, *E. coli* sbm (P27253).

codon of *mcm* is separated by one nucleotide from the start-codon of *meaB* (…CTAGGTTGA…), indicating that all three genes build one transcriptional unit. The deduced protein, MeaB (330 amino acids), shows greatest similarities to ArgK, a member of the LAO transport protein family from *Solibacter usitatus* Ellin6076 (ZP_00519926; identities: 183/298 [61%]; positives: 218/298 [73%]).

The ORF upstream of *epi*, annotated as positive regulator of σ activity, is transcribed in the opposite direction, and the ORF downstream of *meaB* shows no significant similarity to any deposited sequence in the databases, and is separated by 92 nucleotides from the stop codon of *meaB.* Therefore, we concluded that the three-gene operon is responsible for mm-CoA formation in *S. cellulosum* So ce56. Because the genes identified show similar codon usage to *P. putida*, we subsequently intended to verify the in silico annotation by heterologous expression in this strain. A further benefit would be the creation of a host organism suitable for heterologous expression of polyketide biosynthetic gene clusters, requiring mm-CoA as extender unit.

Heterologous Expression of the Operon in *P. putida* and Quantitative Analysis of mm-CoA Formation in the Engineered Strain, FG2005

The three ORFs were subcloned from a BAC in the genomic library of *S. cellulosum* So ce56 [16] onto a minimal p15A replicon by Red/ET subcloning [33, 34]. After PCR amplification of the minimal replicon with oligonucleotides incorporating 50 nucleotides homology to the start and end of the operon, plasmid p15A-amp mutase was generated (Figure 3A). For integration into the *P. putida* chromosome by homologous recombination, homology regions were inserted into the expression plasmid up- and downstream of the operon. This was done by a recombineering technique called triple recombination involving two linear PCR fragments and the circular

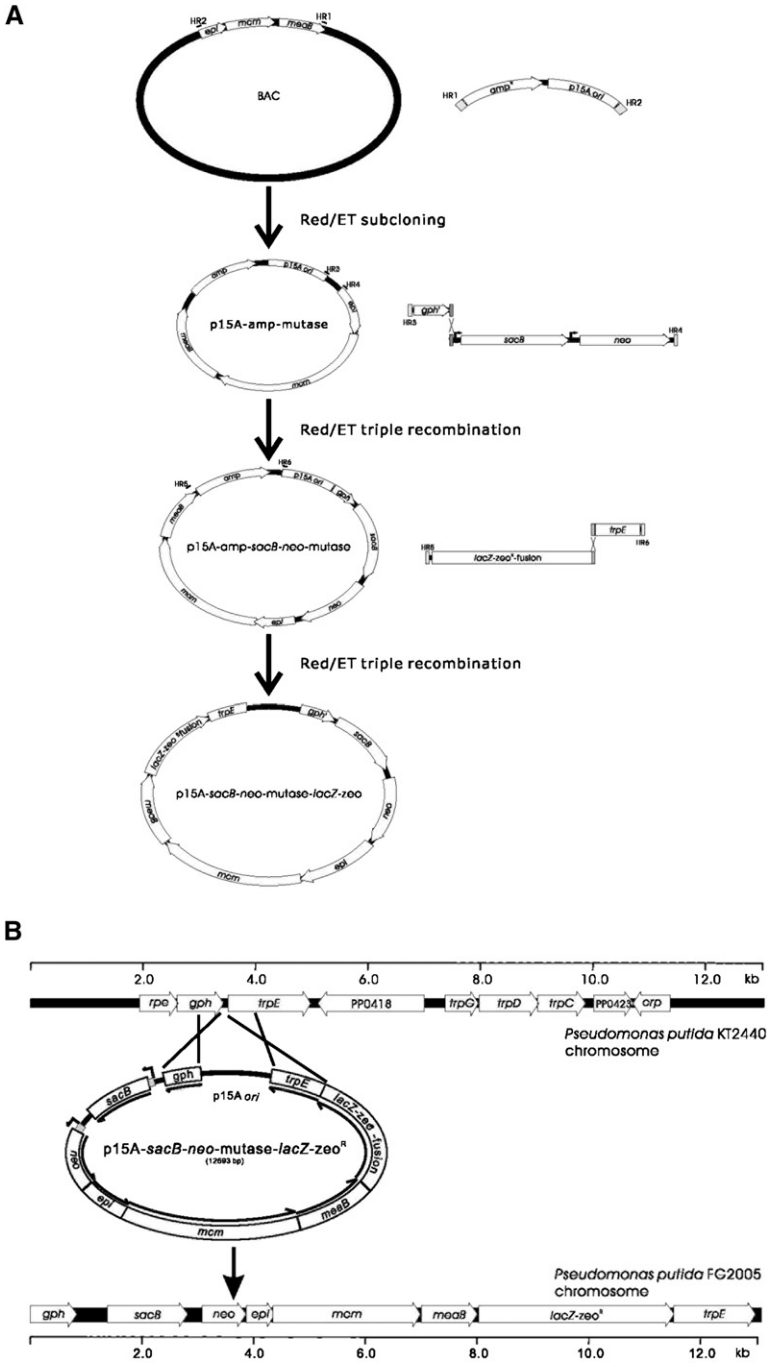

Figure 3. MCM Pathway Expression Plasmid

(A) Construction by Red/ET subcloning and triple recombination. *gph*, phosphoglycolate phosphatase; *trpE*, anthranilate synthase, component I; *sacB*, levansucrase gene from *Bacillus subtilis*; *neo*, aminoglycoside phosphotransferase from Tn5; *epi*, mm-CoA epimerase; *mcm*, mm-CoA mutase; *meaB*, MCM complex protecting protein; *lacZ*-zeo^R-fusion, β-galactosidase-zeocin resitance fusion.

(B) Integration and genome organization of integrant *P. putida*. (Upper panel) DNA fragment of *P. putida* KT2440 wild-type where expression plasmid integrates; (middle panel) expression plasmid; (lower panel) chromosomal DNA fragment after integration of expression plasmid. *rpe*, ribulose-phosphate 3-epimerase; PP0418, lipase, GDSL family; *trpG*, anthranilate synthase, component II; *trpD*, anthranilate phosphoribosyl-transferase; *trpC*, indole-3-glycerol phosphate synthase; PP0423, conserved hypothetical protein; *crp*, catabolite gene activator Crp.

plasmid to be modified [35]. One PCR fragment employed represents the homology region to the *P. putida* chromosome, and the second PCR fragment is a selectable marker. The resulting plasmid, p15A-*sacB*-*neo*-mutase-*lacZ*-zeo, was constructed in two triple recombineering steps (Figure 3A). Each recombination step was verified by restriction analysis (data not shown). The expression plasmid was introduced by electroporation into *P. putida* KT2440, and kanamycin-resistant colonies were selected for further analysis. The integration of the DNA fragment harboring the mm-CoA biosynthetic genes was verified with colony PCR. Four primer pairs amplifying fragments distributed over the whole length of the integrated fragment were used (data not shown). From the 22 analyzed clones, 15 turned out to represent single crossover events, whereas 7 clones resulting from double crossover were identified (data not shown). The organization of the resulting chromosomal region in these double crossover integrants is depicted in Figure 3B.

It was not possible to perform an analysis of the engineered *P. putida* strains for mm-CoA production with the method described by Murli et al. [36] by labeling the acyl-CoA pool in the cell via ^{14}C-labeled β-alanine. Wild-type and recombinant *P. putida* cultures rapidly converted β-alanine into the gas phase (Figure 4). The

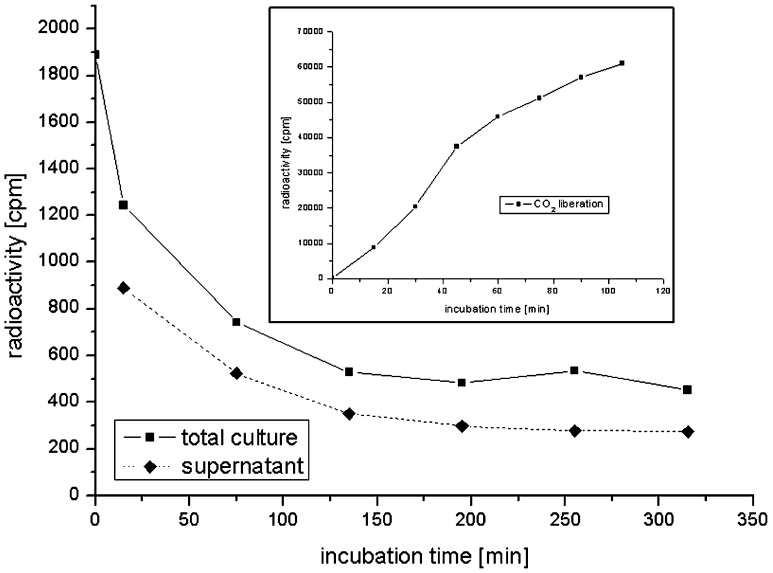

Figure 4. ^{14}C-Labeled β-Alanine Feeding to *P. putida* FG2005 Cultures

Metabolization of the radioactive label. Inset: $^{14}CO_2$ liberation from the culture.

emitted CO_2 of the culture was trapped with KOH solution in a special culture vessel and subsequently analyzed. The emission of labeled CO_2 was in accordance with the loss of radioactivity in the cells and the supernatant (Figure 4, inset).

It was thus necessary to develop a new method for mm-CoA analysis. We chose to use a GC/MS method based on the procedure described by Salanitro and Muirhead [37], in which methylmalonate and its CoA ester are converted into the dibutyl ester (diBE) of methylmalonic acid (mm) after extraction. Methylmalonic acid dibutyl ester (mmdiBE) was then quantitatively analyzed by GC/MS based on an isotope dilution assay with an authentic reference standard, which enabled detection of mm down to 1 nmol per sample (see Experimental Procedures). The double crossover strain, FG2005, was analyzed for mm-CoA production during the growth phase in duplicates in a time course (Figure 5). mm-CoA content reached a stable level of approximately 0.66 nmol/OD unit after 80 hr of incubation (Figure 5). No mm-CoA could by detected in any sample derived from the wild-type. The mm-CoA production of *P. putida* FG2005 was also tested in PMM, a minimal medium, with succinate, the substrate of the MCM reaction, as sole carbon source. Under these conditions, mm-CoA was not detectable (data not shown).

Production of the mm-CoA-Dependent Myxobacterial Secondary Metabolite, Myxothiazol, in *P. putida* FG2005

To test FG2005 for heterologous production of a secondary metabolite that requires mm-CoA, an integrative expression vector with the myxothiazol biosynthetic gene cluster [24, 38] was introduced into *P. putida* FG2005 by conjugation. Briefly, the myxothiazol biosynthetic gene cluster [39] was reconstituted from two cosmids on a single copy vector. Then, the inducible Pm promoter was inserted in front of the operon, and all genetic elements required for transfer and integration into *P. putida* were added by recombineering. The complete integration of the myxothiazol biosynthetic genes into *P. putida* was verified by colony PCR, amplifying parts of *mtaB*, *mtaE*, and *mtaG* (data not shown), and positive clones were designated *P. putida* FG2005::Pm-*mta*. Several clones were subsequently analyzed for production by LC-MS in comparison to *P. putida* KT2440 wild-type and *P. putida* KT2440 harboring the myxothiazol biosynthetic gene cluster as control experiments. Myxothiazol A was identified by comparison to authentic reference standard. Only extracts from *P. putida* FG2005::Pm-*mta* were positive for myxothiazol formation (Figure 6), at a yield of 4.5–5.0 μg/l. Rational optimization of the production with different media and supplements increased the yield significantly. Based on the facts that vitamin B_{12} is needed as cofactor for MCM, and that isovaleryl-CoA is employed as the starter unit of myxothiazol biosynthesis, it seemed likely that the availability of these metabolites limits production. Consequently,

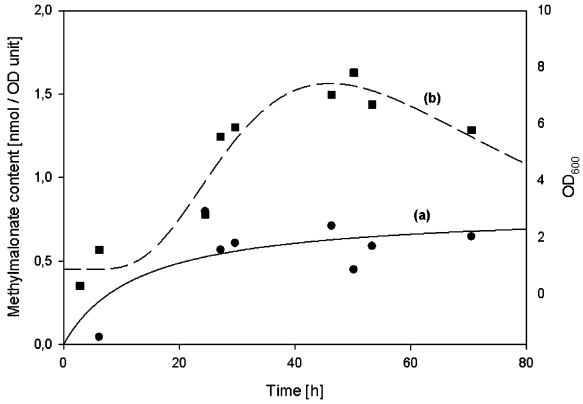

Figure 5. Methylmalonate Detection in a *P. putida* FG2005 Culture with GC/MS

(a) Methylmalonate content in the culture at given time points per OD unit; concentration for the whole extract was calculated from the value of the sample, then divided by the OD_{600} value. Closed circles, mean of 2 measurements.

(b) Cell density of the culture in OD_{600} units at the respective time points. Closed squares, single measurement of OD.

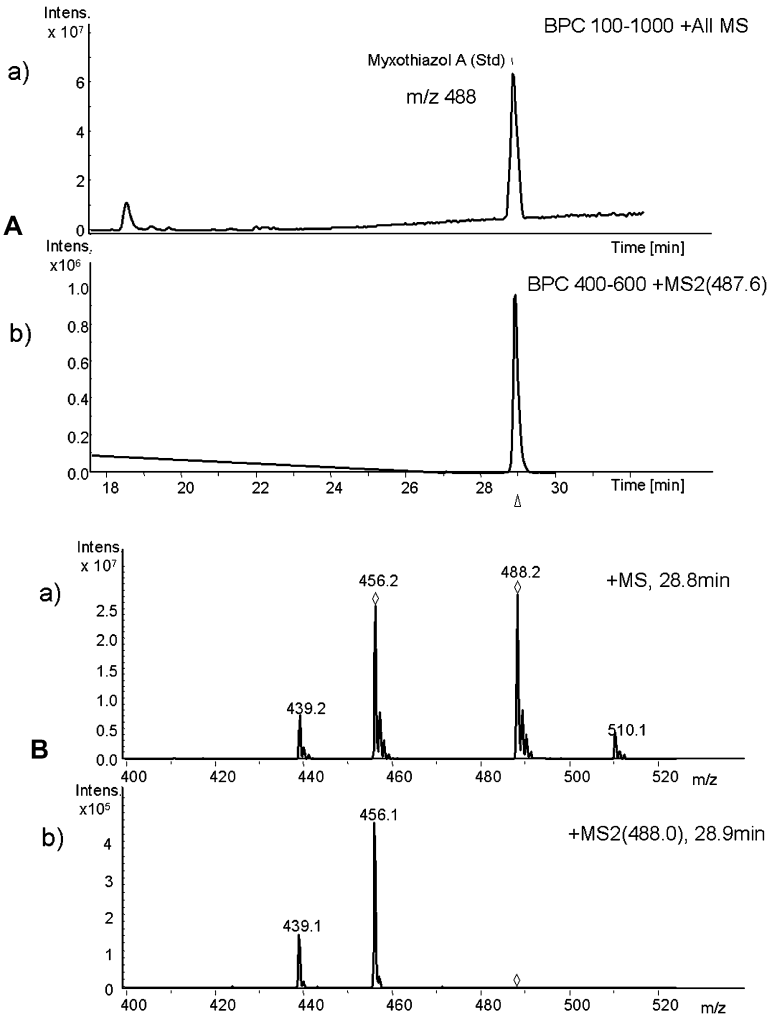

Figure 6. Heterologous Production of Myxothiazol in *P. putida* FG2005::Pm-*mta*

(A) HPLC chromatograms of (Aa) HPLC-MS base peak chromatogram (BPC) of the reference substance, and (Ab) BPC displaying intensity of fragments generated from parent ion of *m/z* = 400–600 in extracts of *P. putida* FG2005::Pm-*mta*.

(B) mass fragmentation pattern. (Ba) Mass spectrum of reference substance myxothiazol A; (Bb) fragmentation pattern generated from peak at 28.9 min in (Ab).

a minimal medium with addition of L-leucine and vitamin B_{12} was used to cultivate *P. putida* FG2005::Pm-*mta*, resulting in a production level of 600 μg/l (data not shown).

Discussion

Natural products are a valuable source of compounds with biological activities [4], and new resources for such compounds are gaining attention. A prerequisite for the exploitation of resources, such as "silent" gene clusters from sequenced genomes or metagenomic libraries, is the availability of heterologous expression systems [5, 9, 40, 41]. From heterologous expression of known biosynthetic gene clusters in *E. coli*, *S. lividans*, *P. putida*, and *B. subtilis*, it is evident that each system has limitations, so that successful expression has only been achieved with gene clusters from closely related microorganisms [40]. Streptomycetes and related actinomycetes are the most widely used systems for heterologous expression [42] because they provide the whole machinery for polyketide synthesis, and most known secondary metabolite biosynthetic gene clusters originate from this genus [5]. The use of *E. coli*, favored because of the existing genetic tools, fermentation knowledge, and ease of handling, requires a lot of strain engineering [10, 43], and there is still the disadvantage of the different G+C content compared with that of most biosynthetic gene clusters. The gap between the more laborious streptomycetes and *E. coli* was recently filled with the heterologous expression of the myxobacterial natural product, myxochromide, in *P. putida*. A PKS/NRPS hybrid molecule was produced in *P. putida* in a 5-fold higher yield in comparison to the natural producer, while fermentation time was reduced 3-fold [11]. Pseudomonads combine the advantages of *E. coli*, like easy handling, genetic tools, and comparable growth rates, with the properties of streptomycetes and myxobacteria, like high G+C content and their ability to produce secondary metabolites. In addition, they express a broad-range phosphopantetheinyl transferase for the posttranslational activation of the carrier protein [12, 44], which is lacking in *E. coli*. However, both organisms fail to produce mm-CoA, an extender unit often used in polyketides [15, 30]. An mm-CoA-negative strain can be enabled to synthesize polyketides, which require mm-CoA as an extender unit by expressing MCM and mm-CoA epimerase. The epimerase is necessary, because, according to the published data, only (2S)-mm-CoA is a substrate for PKS. Dayem et al. [43] engineered an *E. coli* strain to produce 6-dEB by inserting the T7 promoter-driven, broad-spectrum phosphopantheinyl transferase, *sfp*

[45], in front of *prpE*, the gene for the propionyl-CoA synthetase and providing on a plasmid the genes for MCM and mmCoA epimerase from *P. freudenreichii*. On a second plasmid, the genes encoding deoxyerythronolide B synthase were introduced into the heterologous host [43]. This way, they provided the starter unit, propionyl-CoA, by feeding propionate to the culture, which is converted by PrpE to the CoA-ester, and the mm-CoA extender units are supplied by the MCM and mm-CoA epimerase.

In order to enable *P. putida* to produce heterologous secondary metabolites, which require mm-CoA for their biosynthesis, the mm-CoA biosynthetic pathway from a myxobacterium was introduced into this strain. An operon consisting of three ORFs was identified in the ongoing sequencing project of *S. cellulosum* So ce56, where the first and second ORF exhibit significant similarities to the mm-CoA epimerase and MCM, respectively, of *P. freudenreichii shermanii*.

MCM is a member of the small family of enzymes using vitamin B_{12} to create a substrate radical to exchange a hydrogen atom with a group attached to the neighboring carbon atom. Native MCM appears as a dimer; however, some are homodimers (e.g., Sbm from *E. coli* [30]) and others are heterodimers of homologous subunits (e.g., the mutase of *P. freudenreichii shermanii* [28]).

The expression of the coenzyme B_{12}-dependent MCM from *P. freudenreichii shermanii* in *E. coli* led to the production of mm-CoA; however, the addition of hydroxocobalamin (a precursor in vitamin B_{12} biosynthesis) to the culture medium was necessary [43], because *E. coli* lacks the *cobI* genes, required for cobalamin formation. In contrast, all genes necessary for cobalamin production are present in the genome of *P. putida* KT2440 (http://www.tigr.org/), which favors the heterologous expression of MCM in this strain.

When the putative epimerase was used as the query in a BLASTP search, the best hits were glyoxylases and bleomycin resistance genes, both of which belong to the LGSH lyase family. This family is a member of the vicinal oxygen chelate superfamily, which, in addition to mm-CoA epimerase, encompasses extradiol dioxygenases, and the fosfomycin resitance protein [46, 47]. Members of a superfamily typically share a common structural scaffold, but may catalyze totally different overall reactions [47]. Taking this into account, automated annotations of an ORF as a glyoxylase or bleomycin resistance gene in microbial genome projects should be reviewed carefully.

The deduced peptide sequence of MCM aligned well with sequences in the databases (Figure 2B). A database search for MCMs at UniProt (http://www.ebi.uniprot.org/index.shtml) revealed 284 entries comprising sequences ranging from 1139 to 58 amino acids in length. A total of 19 of these peptide sequences represent proteins with more than 1000 amino acid residues. These peptides contain also an additional ArgK domain. The ArgK protein, annotated as MeaB in *S. cellulosum* So ce56 genome, is present in the operon described here as a separate gene, and not similar to the additional N terminus of MCM (Figure 2B). The MCM of *S. cellulosum* So ce56 represents the largest known mutase without the ArgK domain on the same peptide. This may be due to the fact that the first 150–200 amino acid residues of the peptide chain show a weak similarity (around 30% identity) to MCM small subunits. The impact of this N-terminal extension on the function of the enzyme is not clear at present.

The third gene in the operon, *meaB*, was initially identified as a homolog to ArgK of *E. coli*, an ATPase enzyme belonging to the LAO transport proteins. This annotation was later challenged, since many ArgK homologs cluster with MCM, and participation in the propionyl-CoA pathway was assumed. Recently, MeaB, a homolog in *Methylobacterium extorquens* AM1, was shown to protect the MCM complex from inactivation [48]. It was shown that MeaB is not a reactivation factor for MCM, as it is known for the B_{12}-dependent diol dehydratase and glycerol dehydratase [49, 50]. It was suggested that MeaB protects MCM from suicide inactivation by forming a complex [48] and preventing the observed dissociation of subunits [51]. The bound cofactor in the dissociated subunits is supposed to be exposed to O_2, water, or highly reactive radical intermediates, and the inactive form of MCM is generated [51].

If a gene or a cluster of genes and their respective peptides are subjected to biochemical studies, PCR from the respective extrachromosmal DNA entity or chromosomal DNA is currently used, most often, to clone the ORF into expression vectors. This method has limitations if the DNA fragments to be amplified are of large size, and Red/ET subcloning circumvents these limitations [33]. Large stretches of DNA can be subcloned in a fast and simple way by this method (Figure 3A), and only the regions encompassing the PCR primers have to be sequenced to ensure the fidelity of the primers and the correctness of the recombination. With the applied triple recombination technique in the downstream construction of the integrative expression plasmid, engineering of a DNA molecule becomes even more liberated from constraints of "classical" DNA manipulation technologies. It provides a one-step procedure to introduce a DNA fragment at a desired location in the molecule (Figure 3A).

Once the mm-CoA operon was introduced into *P. putida*, a method for the detection of the compound was needed. The failure of the simple and elegant method described by Murli et al. [36], which records the complete acyl-CoA ester pool in a cell through the labeling of coenzyme A with radioactive β-alanine, was most likely caused by the presence of a β-alanine::pyruvate transaminase, a ω-transaminase, which is not present in *E. coli*. A corresponding gene was found in the genome of *P. putida* KT2440 (PP0596). The encoded protein is part of the reductive degradation pathway of pyrimidines [13]. Malonate semialdehyde, the desamination product of β-alanine, is probably metabolized immediately by the Krebs cycle, and, therefore, the labeled β-alanine added to the culture broth almost exclusively ended up in CO_2. We then developed an alternative isotope dilution assay for the analysis of mm-CoA by GC/MS, based on the conversion of short-chain carboxylic acids into their corresponding butyl ester (BE), as described by Salanitro and Muirhead [37]. There is no need for the expensive, labeled β-alanine, and very sensitive quantitation is possible by the isotope dilution assay. This method is also not affected by the presence of a ω-transaminase, and, therefore, is

applicable to all microorganisms. However, the method is indirect in only detecting derivatized compounds, and not the CoA esters directly, resulting in the inability to distinguish between epimers of mm-CoA.

In a proof-of-principle experiment, the myxothiazol biosynthetic pathway from *S. aurantiaca* DW4/3-1 [24] was introduced into the recombinant strain, *P. putida* FG2005. In contrast to the wild-type, this strain enabled the production of myxothiazol, which validates the in silico annotation of the mm-CoA epimerase, because the extender unit in polyketide biosynthesis is exclusively the (2S)-isomer of mm-CoA [52]. As shown in Figure 1, succinyl-CoA is converted by MCM into the (2R)-isomer of mm-CoA. (2R)-mm-CoA is then racemized into the (2S)-isomer by the epimerase. This means that the enzymes of the introduced *mcm*-operon in *P. putida* FG2005 are functional in the proposed manner because the polyketide myxothiazol was synthesized.

The conversion of propionyl-CoA to succinyl-CoA via the MCM pathway is the most common and best-established pathway [53]. *P. putida* is able to grow on L-valine/L-isoleucine as the sole carbon source; however, we were not able to detect mm-CoA (data not show). Therefore, we assume that pseudomonads use one of the other described pathways not involving mm-CoA [53].

The initial yield of 4.5–5.0 μg/l myxothiazol achieved by heterologous expression in *P. putida* FG2005 after cultivation in LB medium is about half the amount of the reported heterologous production of the myxobacterial secondary metabolite, epothilone, in *E. coli* (~10 μg/l) [54]. However, the required strain engineering and the construction of the expression vector was less laborious. Importantly, the starter unit of myxothiazol biosynthesis, isovaleryl-CoA, is a metabolite derived from the degradation of the amino acid L-leucine [13]. This is likely one reason for the low production yield, because the degradation pathway is only active after induction (e.g., during growth on minimal medium with L-leucine as the sole carbon source). Another possibility for the bottleneck of production is the supply of the required extender mm-CoA, which may be caused by a shortage of the MCM cofactor, vitamin B_{12} (compare with [55]). Consequently, we could show that production in a minimal medium based on L-leucine as a carbon source, with the addition of vitamin B_{12}, increased production more than 120-fold, reaching levels of 0.6 mg/l (typical yields in myxobacterial producers are below 10 mg/l). Experiments aimed at a further increase of the biosynthesis of mm-CoA (e.g., to change the TTG starter triplet of *meaB* into ATG) are under way in our laboratory. An increase of the mm-CoA production level after this change might be possible owing to a better expression of MeaB, which would also strengthen the possibility that this protein protects the MCM enzyme complex from inactivation [48].

Significance

We identified, in silico, in the *Sorangium cellulosum* So ce56 genome, an operon consisting of three genes—methylmalonyl-CoA (mm-CoA) epimerase (*epi*); mm-CoA mutase (*mcm*); and *meaB*—responsible for mm-CoA production. The operon was subcloned and heterologously expressed in *Pseudomonas putida* KT2440, which is unable to produce mm-CoA, an extender unit required for the production of a variety of secondary metabolites. The production of the compound was verified and quantitatively analyzed by an isotope dilution method based on GC/MS analysis of the respective diBE. This method can be applied to all microorganisms, because it is not sensitive to the presence of a β-alanine::pyruvate transaminase, which made the use of a previously published method impossible based on feeding of radioactive β-alanine to label the acyl-CoA pool of the cell. In a proof-of-principle experiment, the biosynthetic gene cluster coding for the myxothiazol PKS/NRPS hybrid, megasynthetase, was heterologously expressed in the *P. putida* strain producing mm-CoA. The engineered *P. putida* strain provides an additional expression system for secondary metabolite production aside from *Escherichia coli* or streptomycetes to explore the possibilities of metagenomes, silent gene clusters, or gene clusters from slow-growing organisms for the production of new and altered metabolites with biological activities. Since the parent strain is well characterized, easy to genetically manipulate, and completely sequenced, it should be possible to improve the strain even more by precise genome engineering.

Experimental Procedures

General cloning procedures and DNA manipulations were carried out as described by Sambrook et al. [56]. Red/ET recombineering was performed in *E. coli* HS996, also known as GeneHogs *E. coli* (ResGen; Invitrogen Corporation, Karlsruhe, Germany). Restriction endonucleases were purchased from New England Biolabs (Frankfurt am Main, Germany) and used according to the instructions of the manufacturer. *P. putida* KT2440 [57] was used as heterologous expression host for the mm-CoA biosynthetic operon. *E. coli* and *P. putida* were normally grown in LB medium [58]. For the analysis involving ^{14}C-labeled β-alanine, *P. putida* FG2005 was grown in M medium [59]. For mm-CoA production and GC/MS analysis, *P. putida* FG2005 was fermented in LB medium, PMM medium [60], or minimal medium (1.5 g/l K_2HPO_4, 0.5 g/l KH_2PO_4, 0.2 g/l $MgSO_4 \times 7H_2O$, 2 g/l NaCl, 1 g/l $(NH_4)_2SO_4$, 25 mM L-valine, 7 mM L-isoleucine). L-leucine was added to this minimal medium in the same concentration as L-Isoleucine. This medium was supplemented with 0.1 mg/l vitamin B_{12} if needed.

Identification and Subcloning of the mm-CoA Biosynthesis Operon in *S. cellulosum* So ce56

The contigs of the preliminary *S. cellulosum* So ce56 genome sequence available at the time of investigation were analyzed with the BLASTP software [26] employing the amino acid sequences of the large and small subunits of MCM and mm-CoA epimerase (P11653, P11652, and AY046899, respectively) of *P. freudenreichii shermanii* as query sequences. One contig was identified harboring all homologs to the query sequences. The insert end sequences of a BAC library of *S. cellulosum* So ce56 [16] were analyzed with BLASTP with the identified contig as query to identify a BAC clone harboring the operon. Three independent clones were identified with this procedure: 1·1 F5, 4 D8 and 1·4 C7. The operon was subcloned on a minimal p15A-replicon with Red/ET recombineering [61]. The minimal replicon was amplified with primers mutasub5 (5′-TGCGCGCCGCGCGGCAGCTGCTGGAGGCCTGGAATCCGGCCGGAAAGCGCTGATCGTGCTGACGCTTCATCAGAAG -3′) and mutasub3 (5′-TCGGGAGGCCGGTCGGGGCTGCAGGGGCCGGCCGTGTCGCGGGGGGCGCCATGCGCTAGCGGAGTGTATACTGG-3′) from plasmid pACYC177 [62], with the respective homology arms (underlined) to the target sequence on the BAC. The amplicon was used in the Red/ET subcloning procedure [33] to yield plasmid p15A-amp-mutase (Figure 3A). The following construction steps for creating an integrative plasmid were carried out in a procedure termed "triple recombination," because three DNA fragments are involved in the

recombination event. In our experiment, a plasmid and two PCR fragments were used; the two PCR fragments overlap by 50 bp. A similar procedure was recently used for a chromosomal promoter replacement in *E. coli* [35]. First a *gph*′-*sacB*-Tn5neo cassette was introduced 5′ to the *mcm* operon. The partial *gph* fragment (phosphoglycolate phosphatase) from *P. putida* (see Figure 3B) was amplified with primers homtrp5 (5′-TGACACCCTCATCAGTGCCAACATAGTAAGCCAGTATACACTCCGCTAGCGC*TTAAG*TGCTGGACCAGATGAAGATCGGTA-3′) and homtrp3 (5′-TCGACCTCATTCTATTAGACTCTCGTTTGGATTGCAACTGGTCTAGAGTAGTCTCGTGGCAAGCAGCAATAG-3′) from genomic DNA, and the *sacB-neo* fragment with primers trpsacB5 (5′-TGCCTGACCTATTGCTGCTTGCCACGAGACTACTCTAGACCAGTTGCAATCCAAACGAG-3′) and trpsacB3 (5′-TGGGTCGGGAGGCCGGTCGGGGCTGCAGGGGCCGGCCGTGTCGCGGGGGGCGCCATGATTAGTTCCTCAGAAGAACTCGTCAAGAAGG-3′) from plasmid pIB279 [63]. Both PCR fragments were coelectroporated into recombineering-proficient *E. coli* HS996/p15A-amp-mutase, pSC101BAD$\gamma\beta\alpha$A [64]. The resulting plasmid was designated as p15A-amp-*sacB-neo*-mutase (Figure 3A). In a second step, a *lacZ*-zeo^R-*trpE*′ cassette was introduced downstream of the *mcm*-operon. The *lacZ*-*zeo*^{R} fusion was amplified with primers laczeom5 (5′-TGCGCGCCGCGCGGCAGCTGCTGGAGGCCTGGAATCCGGCCGGAAAGCGCTGAGGAATCAATCATGGTCGCGAGTAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGAC-3′) and laczeom3 (5′-TAGCCGGCAGCGGCCAGGCGCAGGAATTCTTCGCGGTTCATTCAGTCCTGCTCCTCGGCCACG-3′) from plasmid pGT-IRES-*lacZ*-zeo (unpublished data), and the partial *trpE*-fragment (anthranilate synthase, component I) as second homology region to the *P. putida* genome, with primers lacztrp5 (5′-TGGCCGAGGAGCAGGACTGAATGAACCGCGAAGAATTCCTGCGC-3′) and lacztrp3 (5′-TGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGC*TTAA*GAAGGCCTGATCTTCAGCTGGGTC-3′) from genomic DNA. The priming sites are underlined, and artificially introduced restriction site *AflII* in homtrp5 and lacztrp3 are depicted in italics. The two PCR products were coelectroporated into a recombeneering-efficient *E. coli*/p15A-amp-*sacB-neo*-mutase, pSC101BAD$\gamma\beta\alpha$A [64]. The correct clones were identified by restriction digest after each recombination event. The final construct was designated as p15A-*sacB-neo*-mutase-*lacZ*-zeo (Figure 3A).

The sequence of the described mm-CoA biosynthesis operon is deposited at EMBL nucleotide sequence database, with accession number AM260198.

Integration of the mmCoA Operon into *P. putida* KT2440 Genome

The expression plasmid was transformed into *P. putida* by electroporation, and kanamycin-resistant clones were selected. The electrocompetent cells were prepared as follows: 1.4 ml LB medium in a 1.5 ml reaction tube were inoculated with 30 μl of a saturated overnight culture of *P. putida* KT2440 and incubated for 2 hr at 28°C with shaking on a thermomixer (Eppendorf, Hamburg, Germany). The cells were washed twice with ice-cold water and resuspended after the last washing step in the remaining drop of liquid. A 1 μl aliquot of a plasmid minipreparation of p15A-sacB-neo-mutase-lacZ-zeo was added, and the cell suspension was transferred to a 1 mm electroporation cuvette. The cells were pulsed with a voltage of 1.1 kV in an Eppendorf electroporator model 2510 (Eppendorf); then, 500 μl LB medium was added, the cells transferred to a 1.5 ml reaction tube and incubated for 60 min at 30°C, with shaking for phenotypic expression on a thermomixer. The transformed cells were spread on LB agar containing 15 μg/ml kanamycin and incubated at 30°C overnight. To further verify the clones, four internal primer pairs were used for colony PCR reaction (integrchk1-5: 5′-GGACCAGATGAAGATCGGTA-3′ and integrchk1-3: 5′-TGTTCATCGTTCATGTCTCC-3′; integrchk2-5: 5′-CGACTTCCAGTTCAACATCA-3′ and integrchk2-3: 5′-GATTCGAGCAGGTACGAGTT-3′; mutasechk3-5: 5′-GCTTCGCCCACGTCGCCTACC-3′ and mutasechk3-3: 5′-CGACGATGCCGCGGAGGAGGTT-3′; mutasechk4-5: 5′-CGAGACGGGCGAGGGGAACC-3′ and mutasechk4-3: 5′-CGTCTTGTCGCCGAGGATGCT-3′; data not shown).

Analysis of Clones for mmCoA Production with ^{14}C-Labeled β-Alanine

The uptake of the ^{14}C label into the cells was examined in a 10 ml culture of *P. putida* FG2005 in M medium with 0.1 μCi β-alanine (Amersham, Freiburg, Germany) in a 100 ml Erlenmeyer flask, incubated at 30°C in a water bath with agitation. At indicated time points (Figure 4), two 0.1 ml samples were taken. One sample was centrifuged to separate cells from supernatant, and 50 μl of the supernatant was analyzed with a Beckman LS 6500 scintillation counter (Beckman Coulter, Krefeld, Germany). A 50 μl aliquot of the second sample was analyzed without any further manipulation with the scintillation counter.

The liberation of ^{14}C-labeled CO_2 was measured with 10 ml of an overnight culture of *P. putida* FG2005. The culture was transferred into an Erlenmeyer flask-like vessel, which had a small cylinder at the bottom. A 47 % KOH solution was added to this cylinder to absorb the liberated CO_2. After adding 0.1 μCi β-alanine, the vessel was sealed with a screw cap containing a silicon membrane and incubated at 30°C in a water bath with agitation. Every 15 min, a 50 μl sample of the KOH solution was taken with a Hamilton syringe through the membrane and analyzed in a scintillation counter.

Analysis of Clones for mmCoA Production with GC/MS

A 100 ml LB sample supplemented with 50 μg/ml kanamycin was inoculated 1:1000 with an overnight culture of *P. putida* FG2005, incubated at 30°C and 180 rpm on a Multitron rotary shaker (Infors AG, Bottmingen, Switzerland), and harvested at different time points. The cells were collected by centrifugation. The cell pellets were frozen in liquid nitrogen and stored at −80°C until further processing. For extraction, the pellets were thawed on ice, resuspended in 1–2 ml PBS buffer (PBS tablets; Invitrogen) and the cell lysates were prepared with a French press. After addition of 50 ml methanol, the suspension was incubated 1 hr with agitation and then filtered through a folded paper filter. The methanol buffer mixture was removed in vacuo, and the residue dissolved in 1 ml methanol.

Methylmalonic acid and its CoA ester were transformed into mmdiBE by a procedure based on the method of Salanitro and Muirhead [37]. An aliquot (300 μl) of the extract was transferred to a glass vial (1.8 ml), 10 nmol of the internal standard, methyl-d_3-malonic acid, was added, and the mixture was evaporated to dryness in a vacuum concentrator (Eppendorf), and 400 μl hexane and 100 μl HCl (~3 M) in 1-butanol (Fluka, Taufkirchen, Germany) were added to the dry sample. The vials were capped with Teflon-lined screw caps and incubated at 80°C for 2 hr. After cooling down to room temperature, the reaction mixture was neutralized with 500 μl of an aqueous solution of Na_2CO_3 (6% *m/V*), and the vials were centrifuged to achieve complete phase separation. The upper organic phase was injected into the gas chromatograph. All quantitations were carried out in duplicate unless otherwise stated. Samples were measured on an Agilent 6890N gas chromatograph equipped with a 5973N mass selective detector and a 7683B automatic liquid sampler (Agilent Technologies, Waldbronn, Germany). The stationary phase was an HP-5ms capillary column (0.25 mm × 30 m × 0.25 μm, dimethylpolysiloxane with 5% phenyl rests; Agilent Technologies), and the carrier gas was helium, at a flow rate of 1.5 ml/min. The temperature gradient we used was as follows: 70°C for 5 min (isothermal), heating up to 170°C at 5°C/min, heating up to 300°C at 30°C/min, 300°C for 5 min (isothermal), then cooling down to 70°C at 30°C/min. We used a pulsed, splitless injection mode, injecting 2 μl of sample. For quantitation, the mass detector was configured for single-ion monitoring, scanning ions *m/z* 101, 104, and 105 at a dwell time of 100 ms/ion. The quantitation was based on the ratio of the areas of the ions *m/z* 101 (mmdiBE) and *m/z* 104 (methyl-d_3-malonic acid diBE). Calibration was done with triplicate samples of 1, 2, 5, 10, 20, and 50 nmol methylmalonate, each sample containing 10 nmol methyl-d_3-malonate. Data analysis, calibration, and quantitation were done with Agilent ChemStation software. Based on the values obtained by this method, methylmalonate quantities were calculated in nanomoles per OD unit.

Conjugation of a Myxothiazol Expression Plasmid into *P. putida* FG2005

The integrative myxothiazol gene cluster expression vector [24, 38] was introduced into the chromosome of the *P. putida* KT2440 wild-type as well as into the chromosome of the mm-CoA-generating *P. putida* FG2005 by triparental conjugation, as described previously [65]. The selection was carried out on PMM agar plates

containing either tetracycline (25 μg/ml) for selection of the vector, with the myxothiazol biosynthetic genes, or tetracycline and kanamycin (50 μg/ml) to perform selection for the clones containing myxothiazol genes in the mm-CoA-producing *P. putida* FG2005. The obtained clones were tested by colony PCR (Taq-polymerase; Invitrogen) with myxothiazol-specific primers designated for different parts of the gene cluster (parts of *mtaB*: 5′-GAACGTGGTCGTC TCGGGAG-3′ and 5′-CGAATCACCAGCCCGGAGAC-3′, *mtaE*: 5′-TCAAGCCGGATGAGGTCTAC-3′ and 5′-CTTGGACACGGTATCGA GGT-3′ and *mtaG*: 5′-CTCTTCTTCATGCATCCGAC-3′ and 5′-CCGG TACATCTGAACCTGCT-3′) to verify the integration of the whole biosynthetic gene cluster into the chromosome (data not shown).

Analysis of the Heterologous Myxothiazol Production in *P. putida*

The *P. putida* strains were inoculated with a respective overnight culture (1:100) and incubated in 300 ml flasks containing 50 ml LB medium supplemented with tetracycline (25 μg/ml) and with 2% XAD 16 (Rohm und Haas, Frankfurt am Main, Germany) for 1–2 hr at 30°C with shaking. Myxothiazol production was induced by the addition of toluic acid (5 mM); the culture was transferred to 16°C and incubated for 2–3 days. The cells and resin were harvested by centrifugation and extracted with acetone and methanol. The extracts were evaporated and resuspended in 1 ml methanol; 5 μl of the extracts was analyzed by LC-MS. The chromatographic conditions were: RP column Nucleodur C18, 125 × 2 mm, 3 μm, and precolumn C18, 8 × 3 mm, 5 μm. Solvent gradient (with solvents A [water and 0.1% formic acid] and B [acetonitrile and 0.1% formic acid]) from 5% B at 2 min to 95% B within 30 min, followed by 4 min with 95% B at a flow rate of 0.4 ml/min. The mass was detected in positive ionization mode. Myxothiazol A was identified by comparison to the retention times and the MS data of the reference substance ($[M+H]^+ = 488$) (Figure 6). For quantitative analysis, samples were separated on a gradient starting at 80% B and running to 95% B at 5 min, with elution of myxothiazol A occurring at 3 min. Quantitation was carried out in manual MS^2 mode. Ions of *m/z* $[M+H]^+ = 488$ were collected and subjected to fragmentation. The intensities of characteristic fragment ions *m/z* = 456 and *m/z* = 439 were summed up, and peak integration was carried out utilizing the Bruker QuantAnalysis v1.6 software package. A calibration curve was established from serial dilutions of myxothiazol A down to 0.1 μg/ml. Samples under investigation were diluted as required to fit the dynamic range of the method.

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Accession Numbers

The sequence of the described mm-CoA biosynthesis operon is deposited at the EMBL nucleotide sequence database with accession number AM260198.